Journal of Chromatography A, 1460 (2016) 92–99

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Metabolic profile modifications in milk after enrofloxacin administration studied by liquid chromatography coupled with high resolution mass spectrometry

A. Junza[a,b], J. Saurina[b], D. Barrón[a,*], C. Minguillón[a,*]

[a] Department of Nutrition, Food Science and Gastronomy, University of Barcelona, Avda. Prat de la Riba, 171, 08921 Sta. Coloma de Gramenet, Barcelona, Spain

[b] Department of Analytical Chemistry, University of Barcelona, Martí i Franquès, 1-11, 08028 Barcelona, Spain

ARTICLE INFO

Article history:
Received 1 December 2015
Received in revised form 21 June 2016
Accepted 6 July 2016
Available online 7 July 2016

Keywords:
Enrofloxacin
High resolution mass spectrometry (HRMS)
Metabolomics
Multivariate analysis (PCA)

ABSTRACT

High resolution accurate mass spectrometry (HRMS) operating in full scan MS mode was used in the search and identification of metabolites in raw milk from cows medicated with enrofloxacin. Data consisting of *m/z* features were taken throughout the entire chromatogram of milk samples from medicated animals and were compared with blank samples. Twenty six different compounds were identified. Some of them were attributed to structures related to enrofloxacin while others were dipeptides or tripeptides. Additionally, enrofloxacin was administered in a controlled treatment for three days. Milk was collected daily from the first day of treatment and until four days after in the search for the identified compounds. The obtained data were chemometrically treated by Principal Component Analysis. Samples were classified by this method into three different groups corresponding to days 1–2, day 3 and days 4–7 considering the different concentration profile evolution of metabolites during the days studied. Tentative metabolic pathways were designed to rationalize the presence of the newly identified compounds.

1. Introduction

The administration of antibiotics to animals destined to human consumption, either as prophylactics or as growth promoters, has to be a controlled practice. However, it is widespread in farms. The misuse of these drugs in animals intended for human consumption results in the presence of antibiotic residues and their metabolites in foodstuff. Several authors pointed out that the extended presence of these compounds may contribute to the development and transmission of antibiotic-resistant pathogenic bacteria through the food chain [1,2]. Given their generalized consumption in diversity of cultures and particularly in childhood, this effect may result especially significant when milk and dairy products are considered. Nowadays, more than 6 billion people worldwide are active consumers of such products [3].

Dairy farms produce million tons of milk intended for human consumption. To ensure the safety use/intake of these products, the European Union (EU) has established safe *Maximum Residue Limits* (MRLs) for residues of veterinary drugs in milk and animal tissues destined to enter the human food chain [4].

A number of accurate and sensitive methods exist to detect and quantify quinolones, β-lactams, tetracyclines or macrolides in milk [5–12]. However, studies addressing the identification and determination of unknown metabolites or degradation products from the administered antibiotics are scarce. Some of these studies deal with metabolome modifications after pharmacological treatment with quinolones or β-lactams in poultry, swine, bovine or rats [13–19]. Nevertheless, milk is a particularly sensitive food in this regard. Milk is a complex excretion product where drugs and other xenobiotics are cleared. Therefore, the studies addressing metabolome changes, as a result of an antibiotic veterinary treatment, are of particular interest. Nonetheless, the elucidation of such compounds in milk is not common [20–22].

In this context, LC–MS techniques are the most adequate to tackle the study of unknown compounds occurring at very low concentrations. Concretely, liquid chromatography-high resolution mass spectrometry (LC-HRMS) instruments using time-of-flight analyzers (LC-ToF) have been used to determine the exact mass of the compounds detected. This information is used in combination with that provided by linear ion-trap quadrupole-Orbitrap MS (LQT-Orbitrap MS) which produces MS^n spectra. The study of

* Corresponding authors.
E-mail addresses: dolores.barron@ub.edu (D. Barrón), cminguillon@ub.edu (C. Minguillón).

http://dx.doi.org/10.1016/j.chroma.2016.07.016
0021-9673/

resulting data permits the elucidation of non-targeted structures and the tentative suggestion of degradation pathways for the considered drugs.

Additionally, the emergence of unexpected metabolites or the observation of an altered metabolic profile may constitute a tool to discover possible biomarkers of pathologic states and pharmacological treatments. Moreover, these markers would be of interest to evaluate the quality and safety of milk [23,24]. Studies in this context involve the process and interpretation of complex data obtained by LC–MS. Additionally, metabolomic multivariate chemometric tools are also needed to facilitate the recovery of underlying information. In particular, principal component analysis (PCA) constitutes the most frequently employed method for exploratory studies in order to investigate patterns in sample clustering as a function of a diversity of variables.

The current work is focused on the search and identification of metabolites and TPs in milk coming from cows suffering from mastitis and treated with enrofloxacin (ENR). Milk samples found positive to ENR in a random control screening performed by a local control agency (*Laboratori Interprofessional Lleter de Catalunya*—ALLIC, Profession-overlapping Dairy Association of Catalonia) were analysed in the search for possible metabolites produced from the antibiotic. To consider only residues existing in medicated animals, the analytical results were compared with those obtained in the analysis of milk samples from non-treated cows.

The final aim of the study was to identify possible markers of antibiotic intake even when the original compound is not present in milk. Consequently, a cow was therapeutically treated in a controlled way and milk samples were collected during seven days of treatment. The application of PCA in this context was performed considering content of ENR, its metabolites and other compounds whose presence, among other factors, may depend on the stage of the therapeutic process in which the sample is taken.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (MeCN), methanol (MeOH), formic acid (HCOOH), sodium dihydrogen phosphate (NaH_2PO_4) and sodium hydroxide (NaOH), all of analytical grade, were purchased from Merck (Darmstadt, Germany). Water was generated by a Milli-Q purification system of Millipore (Billerica, MA, USA). Solution of 0.1 mol L^{-1} sodium dihydrogen phosphate was adjusted to pH 10 with 5 mol L^{-1} NaOH.

Polymeric cartridges Oasis HLB (3 cm^3 60 mg^{-1}) supplied by Waters (Mildford, MA, USA) were used for solid phase extraction (SPE). Membrane filters Ultra free Durapore PVDF 0.22 μm from Millipore were used to filter the extract before injection into LC-LTQ-Orbitrap.

2.2. Sample collection

Milk samples were provided by the *Laboratori Interprofessional Lleter de Catalunya* (ALLIC, Profession-overlapping Dairy Association of Catalonia) and from the farm *La Saireta S.C.P.* (Vallfogona de Balaguer, Lleida, Spain). The former samples were collected in several farms and resulted positive to ENR in a random control screening. These samples were used in the search for the compounds of interest.

The latter samples were from a cow suffering from mastitis and pharmacologically medicated with Enrovet (Group Divasa Farmavic, Vic, Spain) whose active principle is enrofloxacin (ENR). The pharmacological treatment consisted of the intramuscular administration of 5 mg kg^{-1} of drug for three days. These latter milk samples were collected along the three days of drug administration (1DT-3DT) and during the four days after treatment (1PT-4PT). These samples allowed us to study the evolving profiles for the compounds of interest. Milk from a healthy cow, which had never suffered from mastitis, was used as a blank sample. The samples were stored at −20 °C prior to analysis.

2.3. Sample preparation

A validated method for determination of quinolones and β-lactams in cow milk was used for sample preparation [5]. Thus, to 2 g of milk, 0.5 mL of 0.1 mol L^{-1} sodium dihydrogen phosphate solution at pH 10, and 2 mL of water were added. The samples were shaken using a vortex and were subsequently centrifuged at 6000 rpm for 5 min. A SPE extraction was carried out with the supernatant. The SPE cartridges were preconditioned successively with 1 mL of MeOH, 1 mL of water and 1 mL of 0.1 mol L^{-1} sodium dihydrogen phosphate at pH 10. After sample loading, the cartridges were washed with 3 mL of water and eluted with 2 mL of MeOH. The methanolic eluted fraction was evaporated to dryness using a TurboVap LV under N_2 stream. The extract was reconstituted with 200 μL of water and centrifuged at 9000 rpm for 5 min.

2.4. Chromatographic and mass spectrometry conditions

An LC system (Agilent Technologies mod. 1100, Santa Clara, CA, USA) equipped with an autosampler and coupled to an API 3000 triple-quadrupole mass spectrometer (QqQ) (PE Sciex, Framingham, MA, USA) with a turbo ion-spray source in positive mode was used to quantify ENR. The chromatographic separation was carried out on a Zorbax Eclipse XDB-C8 column (5 μm, 4.6 × 150 mm) from Agilent Technologies. A Kromasil C8 (5 μm, 4.6 × 15 mm) precolumn supplied by Akady (Barcelona, Spain) was used. The mobile phase was composed of an aqueous solution of 0.1% HCOOH (solvent A) and MeCN with 0.1% HCOOH (solvent B) at a constant flow rate of 1 mL min^{-1}. The injection volume was 20 μL. The gradient applied was: from 0 to 1 min, 15% B; 4 min, 45% B; 7 min, 56% B; 8.5 min, 15% B; and 11 min, 15% B. The optimized parameters were: capillary voltage 4500 V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units) and drying gas (N_2) was heated to 400 °C and used at 6500 mL min^{-1} flow-rate. The declustering potential (DP), focusing potential (FP) and entrance potential (EP) were 45, 200 and 10 V, respectively. Multiple reaction monitoring (MRM) was used during the detection. Two different transitions were followed for ENR. First transition (360 → 316) was used for quantification and second one (360 → 342) was used for confirmation.

The LC–MS system consisted of an Agilent Technologies 1100 LC equipped with an autosampler and coupled to a 6220 oa-ToF LC/MS mass spectrometer with an electrospray ionization (ESI) source (Agilent Technologies, Santa Clara, CA, USA) working in positive mode. The column and chromatographic conditions were the same as those used for analysis by LC-QqQ. The conditions of ToF were: capillary voltage 4000 V, temperature 300 °C, drying gas (N_2) at a flow rate of 9 L min^{-1}, nebulizer gas (N_2) 40 psi, fragmentor voltage 150 V, skimmer voltage 60 V and OCT 1 RF voltage 250 V. ToF-MS mass resolving power was approximately 10,000 FWHM at m/z 922. Spectra were acquired over the m/z 50–1100 range. Data storage was performed in profile and centroid modes.

An Accela LC system coupled to an LTQ Orbitrap Velos mass spectrometer, from Thermo Scientific (Hemel Hempstead, UK), with an ESI source in positive mode was used in the elucidation of metabolites. Metabolites and degradation products were separated on a Pursuit UPS C18 column (2.4 μm, 2 × 50 mm) from Varian (Harbor